Isolation of recombinant antibody fragments (scFv) by phage display technology for detection of almond allergens in food products

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A B S T R A C T

Tree nut allergies are considered an important health issue in developed countries. To comply with the regulations on food labeling, reliable allergen detection methods are required. In this work we isolated almond-specific recombinant antibody fragments (scFv) from a commercial phage display library bypassing the use of live animals, hence being consistent with the latest policies on animal welfare. To this end an iterative selection procedure employing the Tomlinson I phage display library and a crude almond protein extract was carried out. Two different almond-specific scFv (named PD1F6 and PD2C9) were isolated after two rounds of biopanning, and an indirect phage ELISA was implemented to detect the presence of almond protein in foodstuffs. The isolated scFvs demonstrated to be highly specific and allowed detection of 40 ng mL^{-1} and 100 ng mL^{-1} of raw and roasted almond protein, respectively. The practical detection limit of the assay in almond spiked food products was 0.1 mg g^{-1} (110–120 ppm). The developed indirect phage ELISA was validated by analysis of 92 commercial food products, showing good correlation with the results obtained by a previously developed real-time PCR method for the detection of almond in foodstuffs. The selected phage clones can be affinity maturated to improve their sensitivity and genetically engineered to be employed in different assay formats.

Keywords:
Almond (*Prunus dulcis*)
Phage display
scFv
ELISA
Allergen labeling
Food allergen

1. Introduction

Almond (*Prunus dulcis*, family Rosaceae) is a tree nut used as ingredient in a wide variety of food products due to its desirable organoleptic attributes, claimed health benefits, and prebiotic properties (Jenkins, Hu, Tapsell, Josse, & Kendall, 2008; Kamil & Chen, 2012; Kendall, Josse, Esfahani, & Jenkins, 2010; Mandalari, Nueno-Palop, Bisignano, Wickham, & Narbad, 2008). However, almond allergens can trigger adverse immune responses in sensitized individuals, including severe anaphylactic shock reactions (Costa, Mafra, Carrapatoso, & Oliveira, 2012; Teuber, Comstock, Sathe, & Roux, 2003). To prevent health-threatening situations, authorities strongly recommend allergic consumers the total avoidance of the offending food. However, unintended exposure to almond-containing products can happen because of mislabeling of food products, cross-contamination during food processing, or undeclared presence of almond in a ingredient. In order to minimize the accidental ingestion of allergenic foods, labels must provide accurate information concerning the list of ingredients. Several countries have enacted labeling guidelines to safeguard allergic individuals (European Parliament and Council, 2011; Regulations Amending the Food and Drug Regulations, 2011). Nevertheless, enforcement of these regulations depend on the availability of sensitive and specific analytical methods that would permit to verify the accuracy of labels.

Current methods for almond detection include mainly DNA-based and immunochemical techniques. Food labeling regulations do not require the detection of allergenic proteins but the allergenic ingredient itself. According to this, DNA represents a more stable

target molecule than proteins, and it is not influenced by geographical or seasonal variations that affect protein composition (Poms, Klein, & Anklam, 2004). However, despite its high sensitivity, the use of DNA analysis in allergen detection is controversial, since it does not directly detect the allergen or any specific protein (Lau, 2014; Poms et al., 2004). Immunochemical techniques are based on the interaction between an antibody and an antigen, which can be an allergen or a marker protein. The enzyme-linked immunosorbent assay (ELISA) is the most widely used technique for routine screening of allergens in foods, and up-to-date ELISA assays have been developed to detect almond proteins (Acosta, Roux, Teuber, & Sathe, 1999; Ben Rejeb, Abbott, Davies, Cléroux, & Delahaut, 2005; Hlywka, Hefle, & Taylor, 2000; Su et al., 2013). Although assays may differ in format, all of them have in common the use of antibodies raised in animals.

Phage display technology provides an alternative to traditional antibody production, allowing the generation of large quantities of affinity probes with a well defined and constant amino acid sequence. Moreover, it permits to bypass of the use of live animals, hence being consistent with the latest policies on animal welfare (European Parliament and Council, 2010). Phage display technology employs libraries of recombinant bacteriophages that expose on their surface functional antibody binding sites, and it allows the isolation of recombinant antibodies with the desirable binding affinity against the antigen by an iterative selection procedure (Clementi et al., 2012). Due to its versatility, phage display technology has multiple applications (Bratkovič, 2010). Regarding food science, phage display technology has been successfully applied to monitor the presence of *Salmonella typhimurium* in food (Sorokulova et al., 2005) or to detect spores of *Clostridium tyrobutyricum* in milk (Lavilla et al., 2010). For it does not depend on animal immunization, it can be used to obtain specific antibody fragments against toxic or allergenic compounds. Using this technology, Lauer, Ottleben, Jacobsen, and Reinard (2005) isolated a scFv against fumonisin B1, a highly toxic mycotoxin found in corn based products, and Garet, Cabado, Vieites, and González-Fernández (2010) reported the isolation of four scFv to detect palytoxin, a marine biotoxin, in shellfish. Phage display technology has also been used to isolate specific scFv that allow the detection of *Cryptosporidium parvum* sporozoites in water and foodstuff (Boulter-Bitzer, Lee, & Trevors, 2009).

The aim of this work was to isolate specific recombinant antibodies against almond proteins employing a phage-display library of single chain variable antibody fragments (scFv), and to develop a phage-ELISA to detect almond proteins in commercial food products using the isolated affinity probes.

2. Materials and methods

2.1. Materials and chemicals

Tomlinson I library, M13KO7 helper phage, and *Escherichia coli* TG1 strain (K12 Δ *(lac-proAB) supE thi hsdD5/F' traD36 proA$^+$B lacI^q lacZΔM15*) were obtained from Source BioScience (Nottingham, UK). The library has a size of 1.47×10^8 pfu mL^{-1}, and is based on a single human framework for VH (V3-23/DP-47 and JH4b) and Vk (O12/O2/DPK9 and Jk1), which encodes the most common human canonical structure. ScFv is displayed fused with the terminal phage gene III protein, and it is cloned in the ampicilin resistant phagemid vector pIT2.

Almonds "Marcona" cultivar and other tree nuts were acquired from local retailers in Madrid (Spain). After being shelled, they were stored at −20 °C until further use. A total of 13 different almond varieties (Table 1) kindly provided by the IRTA (Institut de Recerca i Tecnologia Agroalimentàries, Tarragona, Spain) and the CEBAS-CSIC (Centro de Edafología y Biología Aplicada del Segura-Consejo Superior de Investigaciones Científicas, Murcia, Spain), were analyzed to check the specificity of the assay. Animal and plant species —including representative members of the Rosaceae family— employed in specificity assays (Table 2) were purchased in different local markets in Madrid. Finally, a total of 92 commercial food products were purchased from different retail markets and delicatessen stores (Spain).

Table 1
Phage-ELISA results (OD 450 nm) obtained for different almond kernel cultivars with scFvs from clones PD1F6 and PD2C9.

Prunus dulcis cultivars	PD1F6	PD2C9
Almond "Marcona"	4.05 ± 0.05	3.96 ± 0.06
Almond "Bitter"	3.69 ± 0.19	3.63 ± 0.16
Almond "Guara"	4.37 ± 0.20	4.55 ± 0.03
Almond "Glorieta"	4.15 ± 0.21	4.13 ± 0.25
Almond "Ferragnes"	3.68 ± 0.14	3.53 ± 0.06
Almond "Vairo"	4.39 ± 0.17	4.38 ± 0.16
Almond "Francoli"	4.12 ± 0.12	4.03 ± 0.15
Almond "Masbovera"	3.33 ± 0.10	3.36 ± 0.17
Almond "Belona"	4.45 ± 0.10	4.28 ± 0.06
Almond "Marinada"	3.64 ± 0.19	3.57 ± 0.20
Almond "Tarraco"	4.42 ± 0.14	4.41 ± 0.24
Almond "Constanti"	4.07 ± 0.08	4.00 ± 0.09
Almond "Soleta"	4.49 ± 0.10	4.49 ± 0.07
Almond "Marta"	4.25 ± 0.17	4.22 ± 0.22

HRP/anti-M13 monoclonal mouse antibody was purchased from GE Healthcare (München, Germany). Tryptone, yeast extract and European bacteriological agar were purchased from Laboratorios Conda (Madrid, Spain). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

PBS composition is 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4. The protein extraction buffer consisted of 0.035 M phosphate solution containing 1 M NaCl, pH 7.5. 2xTY broth is 16 g L^{-1} tryptone, 10 g L^{-1} yeast extract and 5 g L^{-1} NaCl. TYE agar is 15 g L^{-1} bacto-agar, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 8 g L^{-1} NaCl.

2.2. Preparation of protein extracts

All samples (Tables 1 and 2) were ground using a mortar and pestle, and stored in screw-capped vials at −20 °C. Protein extracts were prepared by adding 200 mg of sample to 1800 μL of protein extraction buffer. After shaking for 10 min at room temperature in a vertical rotator (HulaMixer Sample Mixer, Life Technologies, Carlsbad, CA) to extract soluble proteins, the slurry was centrifuged at 10,000 *g* for 10 min at 4 °C, and the supernatant was filtered through a 0.45 μm syringe filter (Sartorius, Göttingen, Germany). Bicinchoninic acid (BCA) assay (Thermo Fisher Scientific Inc., IL, USA) was employed to determine protein concentration. Protein extracts were stored at −20 °C until further use.

2.3. Preparation of binary mixtures

To evaluate the sensitivity of the assay, binary mixtures of almond Marcona in wheat flour (100–0.01 mg g^{-1}) were prepared using a food processor (Thermomix, Vorwerk, Germany). Concentration of 100 mg g^{-1} was prepared by adding 50 g of ground almonds to 450 g of wheat flour. Then, 50 g of the former mixture was added to 450 g of wheat flour (10 mg g^{-1}). Remaining concentrations were made in a similar way. Additional mixtures of 50, 25, 5, and 0.5 mg g^{-1} were prepared by mixing 250 g of wheat flour with 250 g of the mixtures containing 100, 50, 10 and 1 mg g^{-1}

Table 2
List of species not showing cross-reactivity with almond in the indirect phage-ELISA.

Species		
Nuts		
Brazil nut (*Bertholletia excelsa*)	macadamia (*Macadamia integrifolia*)	pine nut (*Pinus pinea*)
cashew nut (*Anacardium occidentale*)	peanut (*Arachis hypogaea*)	pistachio (*Pistacia vera*)
hazelnut (*Corylus avellana*)	pecan nut (*Carya illinoinensis*)	walnut (*Juglans regia*)
Vegetal Species		
anise (*Pimpinella anisum*)	flaxseed (*Linum usitatissimum*)	paprika (*Capsicum annuum*)
apple (*Malus domestica*)	garlic (*Allium sativum*)	pea (*Pisum sativum*)
apricot (*Prunus armeniaca*)	kiwifruit (*Actinidia deliciosa*)	peach (*Prunus persica*)
asparagus (*Asparagus officinalis*)	lentil (*Lens culinaris*)	pear (*Pyrus communis*)
aubergine (*Solanum melongena*)	lupine (*Lupinus albus*)	plum (*Prunus domestica*)
banana (*Musa acuminata*)	maize (*Zea mays*)	pumpkin seed (*Cucurbita maxima*)
barley (*Hordeum vulgare*)	mandarin orange (*Citrus reticulata*)	sesame (*Sesamum indicum*)
carrot (*Daucus carota*)	melon (*Cucumis melo*)	tiger nut (*Cyperus esculentus*)
cherry (*Prunus avium*)	oats (*Avena sativa*)	tomato (*Solanum lycopersicum*)
chickpea (*Cicer arietinum*)	olive (*Olea europaea*)	vanilla (*Vanilla planifolia*)
cinnamon (*Cinnamomum verum*)	onion (*Allium cepa*)	wheat (*Triticum aestivum*)
cocoa (*Theobroma cacao*)	orange (*Citrus sinensis*)	zucchini (*Cucurbita pepo*)
common bean (*Phaseolus vulgaris*)		
Animal Species		
cattle (*Bos taurus*)	poultry (*Gallus gallus domesticus*)	swine (*Sus scrofa domestica*)
fish (*Salmo salar*)		
Others		
Brown sugar	milk	

respectively. Protein extracts from binary mixtures were prepared following the procedure described in Section 2.2.

2.4. Preparation of phage display libraries for biopanning procedure

Phage display library was prepared for selection procedure following the manufacturer's protocol. Briefly, the library was amplified in 200 mL of 2xTY broth containing 100 μg mL^{-1} ampicillin and 1% (w/v) glucose, and it was incubated with shaking (250 rpm) at 37 °C until reaching an optical density of 0.4 at 600 nm (OD_{600}). Then, 2×10^{11} particles of helper phage were added to 50 mL of the former bacterial culture, and the flask was incubated in a water bath at 37 °C for 30 min. Bacterial cells were centrifuged at 3000 g for 10 min at 4 °C, resuspended in 100 mL of 2xTY broth containing 100 μg mL^{-1} ampicillin, 50 μg mL^{-1} kanamycin and 0.1% (w/v) glucose, and incubated with shaking (250 rpm) at 30 °C overnight. Next day, phage particles were precipitated with 20% polyethylene glycol – 6000, 2.5 M NaCl, and pelleted by centrifugation at 3300 g for 30 min at 4 °C. Phage pellet was resuspended in 4 mL of PBS, and centrifuged at 11,600 *g* for 10 min to remove any bacterial debris. Phages were titered, and kept at 4 °C for short term storage or at −80 °C in 15% glycerol for longer term storage.

2.5. Target immobilization

To avoid the isolation of unspecific phages which would produce false-positive results (Menendez & Scott, 2005), polystyrene paddles and magnetic beads were alternately used as coating surface. During the first round of selection, polystyrene paddles (Nunc, Denmark) with a surface area of 5.2 cm^2 were coated with 100 μg mL^{-1} of almond extract in PBS (positive screening) or with 100 μg mL^{-1} of peanut extract (negative screening), and incubated overnight at 4 °C. Previously to perform the experiment, paddles were blocked with 3% BSA at 37 °C for 1 h. For the second round of selection, proteins were coupled to Dynabeads M−280 Tosylactivated (Invitrogen, Life Technologies, Carlsbad, CA, USA), following manufacturer's instructions. Briefly, 5 mg of Dynabeads were coated with 100 μg of almond proteins (positive panning) in 0.1 M Na-phosphate buffer, pH 7.4, to a final volume of 150 μL and then, 100 μL of 3 M ammonium sulphate in Na-phosphate buffer was added. Coupling procedure was performed on a vertical rotator at 37 °C overnight. Next day, Dynabeads were blocked with 1 mL of 0.5% (w/v) BSA in PBS, 1 h at 37 °C with rotation. The same procedure was performed with the dynabeads used for negative panning, but employing a peanut protein extract as the ligand.

2.6. Selection of scFv clones against almond

Approximately 10^{12} phage particles from Tomlinson I library in 2 mL of 3% (w/v) BSA in PBS were added to the peanut-coated polystyrene paddle and it was then incubated at 25 °C for 60 min on a rotator. After negative panning, supernatant containing unbound phage particles was added to the almond coated paddle, and it was incubated at 25 °C for 60 min with rotation, and for further 60 min without rotation. Unbound phages were removed by washing 10 times with PBS, and specifically bound phages were eluted by adding 500 μL of trypsin solution (1 mg/mL trypsin in PBS) for 10 min at room temperature with rotation. A total of 250 μL of the former solution was employed to infect 1.75 mL of a TG1 cell culture at an OD_{600} of 0.4. After incubating 30 min at 37 °C in a water bath, infected cells were spread on a TYE agar plate containing 100 μg mL^{-1} ampicillin and 1% (w/v) glucose, and grown overnight at 37 °C. Titre of eluted phage was also determined. Next day, *E. coli* colonies were scraped into 2 mL of 2xTY containing 15% glycerol and stored at −80 °C. To amplify the phages for the second round of selection, 50 μL of recovered bacteria from the first panning experiment were inoculated into 50 mL of 2xTY containing 100 μg mL^{-1} ampicillin and 1% (w/v) glucose, and incubated at 37 °C until reaching an OD_{600} of 0.4. Then, 10 mL of the culture was infected with 5×10^{10} particles of helper phage. Phage particles were rescued as described above. A second round of selection was performed as described above, but employing 2.5 mg of Dynabeads instead of polystyrene paddles, and increasing the number of washes to 20.

2.7. Polyclonal indirect Phage-ELISA

The enrichment of the phage display library with almond-binding phages was assessed through polyclonal phage-ELISA: (1) Flat-bottom polystyrene microtiter plates (F96 MaxiSorp Nunc immuno plates, Nunc, Denmark) were coated with 30 μg mL^{-1} of almond extract, peanut protein extract (30 μg mL^{-1}) or a BSA solution (100 μg mL^{-1}), in PBS; (2) microtiter plates were washed 3 times and blocked with 0.1% gelatin in PBS for 1 h at 37 °C; (3) after washing 3 times, 10 μL of precipitated phages from each round of selection (containing approximately 10^{10} phage particles) was added to 100 μL of 1% ovalbumin in PBS, and plates were incubated for 1 h at room temperature; (4) after washing 10 times, plates were incubated at room temperature for 1 h with 100 μL of a 1:5000 dilution of a HRP/anti-M13 monoclonal mouse antibody in 1% ovalbumin; (5) finally, plates were washed 5 times, and 100 μL of tetramethylbenzidine substrate solution was added to each well, and plates were incubated in the dark. Color development was performed for 10 min at room temperature and the reaction was stopped with 1 M sulphuric acid. OD_{450} was measured with an iEMS Reader MF (Labsystems, Helsinki, Finland). All washing steps were performed with PBS. All experiments were performed in triplicate.

To assess the ability of single clones to recognize almond proteins, 150 individual colonies from the second round of selection were randomly picked. Single colonies were inoculated in cell culture microplates (Nunc, Denmark) containing 100 μg mL^{-1} ampicillin and 1% glucose, and plates were grown overnight at 37 °C with shaking (250 rpm). Next day, 5 μL from each well was used to infect a second microplate containing 200 μL 2xTY, with 100 μg mL^{-1} ampicillin and 1% glucose. Plates were incubated for 2 h at 37 °C before adding 10^9 particles of helper phage per well. After 1 h of incubation at 37 °C, the plates were centrifuged at 1800 g for 10 min at 4 °C. Bacterial pellets were resuspended in 200 μL 2xTY containing 100 μg mL^{-1} ampicillin, 50 μg mL^{-1} kanamycin and 0.1% glucose, and incubated overnight at 30 °C. Next day, plates were centrifuged at 1800 *g* for 10 min, and supernatants were employed in monoclonal phage ELISA. To that end, immuno plates were coated overnight with 30 μg mL^{-1} of almond protein extract (positive control) or with 30 μg mL^{-1} of peanut protein extract (negative control) in PBS. ELISA assay was developed as described above, but employing 50 μL of the phage supernatant diluted in 100 μL of 1% ovalbumin instead of precipitated phages.

2.8. Sequence analysis

Phagemid DNA from almond-recognizing clones was amplified by PCR using GoTaq Green Master Mix (Promega, Madison, USA). PCR was carried out to check clone from single colonies for the presence of full length VH and Vκ inserts with the following PCR program: 95 °C for 9 min, then, 95 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s for 30 cycles, and final extension at 72 °C for 7 min. Primers used were *LMB3* (5′-CAG GAA ACA GCT ATG AC-3′) and *pHEN seq* (5′-CTA TGC GGC CCC ATT CA-3′) for the whole scFv fragment. *LMB3* and *link seq new* (5′-CGA CCC GCC ACC GCC GCT G-3′) were used to amplify the VH fragment, and *DPK9 FR1 seq* (5′-CAT CTG TAG GAG ACA GAG TC-3′) and *pHEN seq*, to amplify the Vκ fragment. PCR products were examined by electrophoresis on 2% agarose gel.

Phagemid DNA from the clones that presented a complete VH + Vκ fragment was amplified with primers *LMB3* and *pHEN seq*, employing Velocity DNA Polymerase (Bioline, UK), with the following PCR program: 98 °C for 2 min, then, 98 °C for 30 s, 55 °C for 30 s, 72 °C for 15 s for 30 cycles, and final extension at 72 °C for 5 min. PCR products were loaded in a 1.5% low melting agarose gel (Laboratorios Conda, Spain), containing Gel Red (Biotium, Hayward, CA) in Tris acetate-EDTA buffer. DNA bands were purified with the QIAquick Gel Extraction kit (Qiagen GmbH, Hilden, Germany) and sequenced at Parque Científico de Madrid (Spain).

Nucleotide sequences were compared using European Molecular Biology Open Software Suite (Emboss software), and then analyzed with IgBLAST to determine framework and complementary determining regions (CDR) of the VH and Vκ chains. Amino acid sequences were deduced from the nucleotide sequences by Expasy website (www.expasy.org).

2.9. Monoclonal almond phage ELISA

The almond-phage ELISA was performed essentially as described in Section 2.7 with the following modifications: All protein extracts assayed (heterologous species, wheat flour/almond binary mixtures, and commercial samples) were prepared following the procedure described in Section 2.2., and they were diluted 1:200 in PBS. Multi-well plates were coated with 100 μL of the diluted extracts, and incubated at 4 °C overnight. Then, the plates were washed with PBS and the wells blocked with 0.1% gelatin at 37 °C for 1 h. Following another washing step, 100 μL of 1% ovalbumin in PBS, containing $3 \cdot 10^8$ PEG-NaCl precipitated phage particles of the selected almond-specific clone (PD1F6 or PD2C9) were added to each well. The plates were incubated at 37 °C with shaking for 1 h before being washed 3 times with PBS. The remaining steps were performed as described in Section 2.7. A standard curve of wheat flour/almond binary mixtures (100–0.01 mg g^{-1}) was included in each plate. A wheat flour protein extract was included as negative control in each plate. To avoid false positives, negative control wells were included for each sample analyzed, where the 1% ovoalbumin did not contain phage particles, in order to check if proteins in the sample cross-reacted with the secondary antibody. Each sample was analyzed in triplicate.

2.10. Assay validation

The specificity of the assay was assessed by challenging the isolated clones to protein extracts obtained from different animal and plant species (Table 2), that had been previously diluted 1:200 in PBS. Each sample was analyzed in triplicate.

The limit of detection (LOD) was calculated following the guidelines of the International Union of Pure and Applied Chemistry (IUPAC) (Thompson, Ellison, & Wood, 2009), considering the minimum concentration of target protein that exhibits an absorbance value larger than the mean absorbance value of the blank (wells with PBS) plus 3 times the standard deviation (SD), after 20 experiments. The LOD for the binary mixtures of wheat flour matrix spiked with almond was also determined, but employing wells coated with wheat flour protein extract as blank.

Almond protein extract was tested in the concentration range from 0 to 30 μg mL^{-1}. The concentration–response curve was obtained by plotting the absorbance values *vs* the protein concentration and the standard curve obtained for almond dilution was fitted to the four-parameter logistic equation using Origin 8.0 software (OriginLab Corp., USA):

$$y = (A - D) \Big/ \left[1 + (x/C)^B\right] + D,$$

where A is the maximum absorbance at infinite concentration, B is the curve slope at the inflection point, C is the x value at the inflexion point, and D is the minimum absorbance for no analyte (background signal).

Data were analyzed for statistical significance by one-way ANOVA and the Fisher's least significant difference (LSD) test ($p < 0.05$) using Statgraphics Centurion 15.2.14 (XV) (Statpoint Technologies, Inc., Warranton, VA).

To determine the effect of heat treatment on the isolated phage-scFvs' ability to identify heat treated almond protein, 70 g of ground almond were processed in an oven at 160 °C for 13 min, and 70 g were autoclaved for 15 min at 121 °C. Assays were performed in a similar way as for raw almond protein.

Additionally, a concentration–response curve was constructed employing binary mixtures of a wheat flour matrix spiked with decreasing concentrations of raw almonds that ranged from 100 mg g^{-1} to 0.01 mg g^{-1}.

The applicability of the almond phage-ELISA was validated through the analysis of 92 commercial processed food products. Samples included breakfast cereals, ice cream, cookies, nut beverages, chocolate, or nutritional bars because those food types could contain, or might have been exposed to cross-contamination with almond. Furthermore, in order to confirm the results, samples were also analyzed by a real-time PCR method developed in our laboratory that employs almond specific primers and probe, and targets the ITS1 gene (López-Calleja et al., 2014).

3. Results and discussion

3.1. Libraries screening

Screening of phage display libraries is usually carried out employing purified molecules (Hoogenboom et al., 1998). If the desirable antigen is not commercially available, a secondary approach can be the purification of the target at laboratory-scale. However, plant proteins are products of multigene expression with many specific post translational modifications, hence purification is generally incomplete and time consuming. As we previously reported, isolation of a Brazil nut specific phage scFv was successfully achieved employing a complete Brazil nut protein extract as target (de la Cruz et al., 2013). Therefore, in order to isolate an almond-specific phage clone from the Tomlinson I phage display library, we decided to use a crude almond extract.

Enrichment of the phage display library in almond-binding clones through the different rounds of selection was tested in an indirect way by measuring the ratio between the input number of phage particles at the beginning of each experiment (which was maintained at 10^{12} pfu mL^{-1}) and the number of phage particles recovered at the end. After the first round of selection, the number of phage particles recovered was 10^5 pfu mL^{-1}, and after the second round this number increased to $2 \cdot 10^8$ pfu mL^{-1}, which implies an increase in the output number of phage particles of about 2000 times after two rounds of affinity selection. This enhancement is in accordance with the expected enrichment of the phage display library with specific phage binders among selection rounds (Lee, Iorno, Sierro, & Christ, 2007). Thus, the results obtained indicated that the Tomlinson I library might be enriched with almond binding phages.

3.2. Polyclonal phage-ELISA

To confirm the suitability of the selection procedure, a polyclonal phage-ELISA was performed with phage pools from the naïve library, and from the first and the second rounds of panning. The observed results —higher absorbance values in wells coated with almond protein than wells coated with peanut protein when employing phages from the second round of selection— (Fig. 1) indicated that phage population that specifically recognized the almond protein had increased compared to the naïve library.

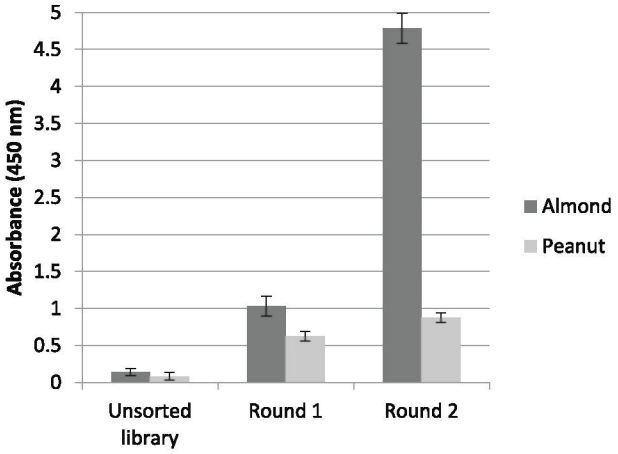

Fig. 1. Indirect phage-ELISA results obtained with polyclonal phages rescued at each round of selection against almond extract. Precipitated phages from each round of selection were analyzed by ELISA. Absorbance values are the mean of three independent determinations. Error bars show the standard deviation for each set of data.

According to this result, additional rounds of selection were not considered necessary.

3.3. Monoclonal phage-ELISA

To isolate and identify the scFvs recognizing almond protein, 150 *E. coli* TG1 colonies infected with phages coming from the second round of selection were randomly picked. A total of 32 out of 150 clones (21%) were selected for binding almond extract and not peanut extract used as negative control (absorbance values against almond/absorbance against negative control >4). Those 32 clones were selected for further analyses.

3.4. Sequence analysis of the positive clones

DNA from the 32 selected clones was amplified with *LMB3* and *pHEN seq* primers, and PCR products were analyzed in agarose gel in search for phage clones holding a complete VH and Vκ insert (VH + Vκ insert, approximately 935 bp). A total of 9 out of the 32 clones analyzed showed a band with the desirable size, and nucleotide sequencing revealed only six different sequences in the clones selected from the Tomlinson I library. One of them, finally named PD1F6, was shared by 4 clones, and the sequences obtained from the remaining 5 clones were all different. Deduced amino acid sequences of scFv from all clones, showing the CDRs and immunoglobulin framework regions (FRs), are illustrated in Fig. 2. After specificity analyses, the best clones, PD1F6 and PD2C9, were selected for an in-depth study.

3.5. Assay specificity and detection limit

Indirect phage ELISA assay using precipitated phage-antibody fragments from clones PD1F6 and PD2C9 was able to detect almond proteins from almond kernels belonging to all different cultivars analyzed (the average absorbance values in ELISA were 4.07 ± 0.36 for PD1F6 clone and 4.03 ± 0.39 for PD2C9 clone), therefore proving the capacity of the assay for detecting diverse almond varieties (Table 1). Absorbance values increased in a concentration-dependent manner, until reaching saturation levels. As heat processing can affect solubility and integrity of almond proteins (Sathe, Teuber, & Roux, 2005), the effect of heat treatment on the phage- ELISA results was tested. As it has been previously described (Venkatachalam, Teuber, Roux, & Sathe, 2002), a

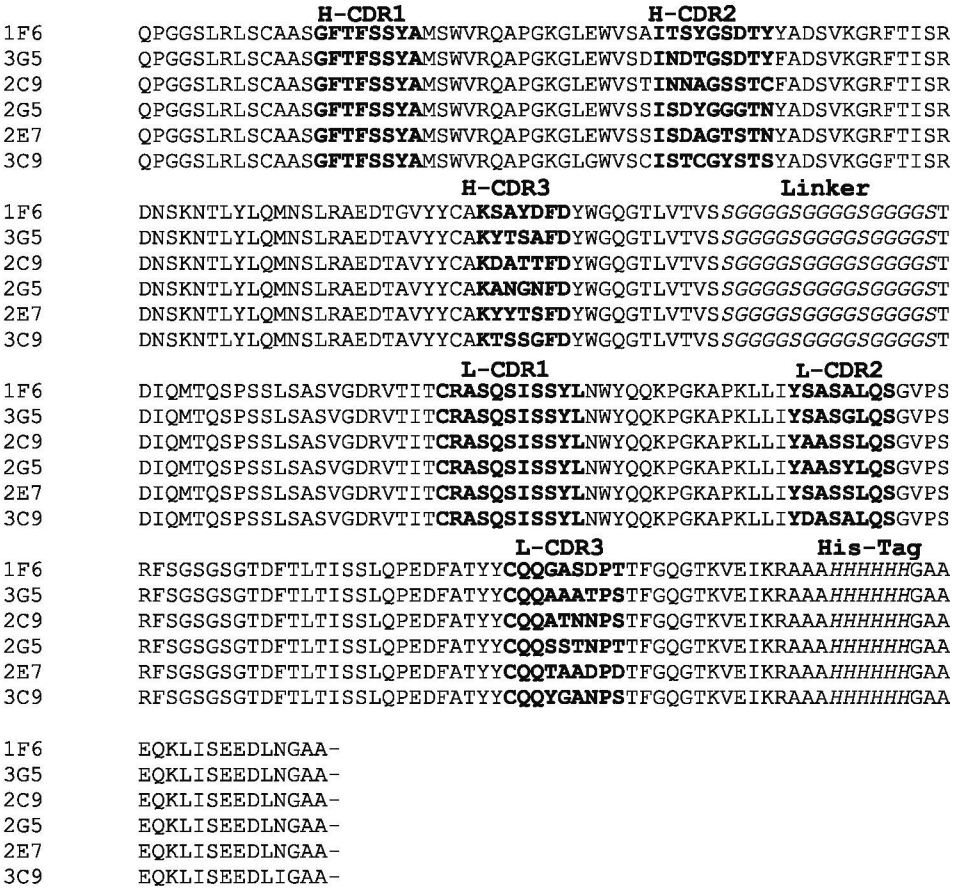

Fig. 2. Amino acid sequences of the almond binding scFvs deduced from the nucleotide sequences by Expasy Web site. Positions of the respective complementary determining regions for the variable domains of heavy (H-CDR 1–3) and light (L-CDR 1–3) chains are indicated.

reduction on protein solubility was observed with the different treatments (protein concentration obtained from raw almond was 8.08 ± 0.82 mg/mL, from roasted almond was 1.45 ± 0.03 mg/mL and from autoclaved almond was 6.24 ± 0.43 mg/mL). However, the developed method still was able to detect heat treated almond proteins (Fig. 3). For PD1F6 clone, the lower concentration that could be reliably detected was of 40 ng mL^{-1} for raw almond, of 70 ng mL^{-1} for autoclaved almond, and of 100 ng mL^{-1} for roasted almond, when protein extracts were diluted in PBS. For PD2C9 clones, the values were 90 ng mL^{-1} for raw, 50 ng mL^{-1} for autoclaved, and 250 ng mL^{-1} for roasted almond.

Cross-reactivity assays using phage-antibodies PD1F6 and PD2C9 showed that none of the non-target protein extracts tested (Table 2) developed an absorbance signal higher than the LOD of almond proteins in PBS. Among the plant species analyzed, edible parts from different members of the *Prunoideae* subfamily (peach, apricot, plum and cherry) were included in order to discard undesirable cross-reactivity reactions due to the hypothetical presence of similar epitopes in these close-related species.

Food matrix components can interfere with the assay performance (Blais, Gaudreault, & Phillippe, 2003), so food matrix effect was included as another factor to consider in the assay. With that purpose, wheat flour was chosen as a representative matrix to mimic the effect of cereal-based products, and binary mixtures of wheat flour spiked with almond were analyzed. Under these conditions, the LOD achieved with the almond-ELISA was of 0.11 mg g^{-1} (110 ppm) for clone PD1F6 and of 0.12 mg g^{-1} (120 ppm) for clone PD2C9 (Fig. 4). However, almond products can be constituted by a large number of food matrices that undergo a wide variety of food processes. Therefore, to quantify the amount of almond protein in a particular sample, it would be necessary to include a standard curve prepared with a food matrix as similar as the analyzed product as possible.

3.6. Determination of almond in commercial food products

The applicability of the indirect phage ELISA method developed for detection of almond proteins in foodstuffs was assessed through analysis of 92 commercial food products (Table 3) that contained or could contain almond in their composition. Among the samples analyzed, 17 were labeled as containing almond as ingredient, 17 labeled as "containing traces of almond", 43 were labeled as "may contain traces of nuts" and the remaining 15 did not declare to contain any nuts. Almond protein was detected in 13 of the 17 processed foods that included almond in the ingredient list. Regarding the 4 samples that did not show positive results in ELISA, real-time PCR analysis supported negative results for the two chocolate samples, but amplified almond DNA from the two almond beverage samples. Lack of detection of almond protein or almond DNA in the chocolate samples might be due to a fraudulent substitution by other nuts, as real-time PCR is consistent with ELISA results. However, the absence of positive ELISA results in the beverage samples can be attributable to epitope denaturation during the UHT process. Almond milk is a colloidal dispersion obtained after the heat treatment of ground almonds with water. Mills, Jenkins, and Bannon (2003) argue that proteins will show an

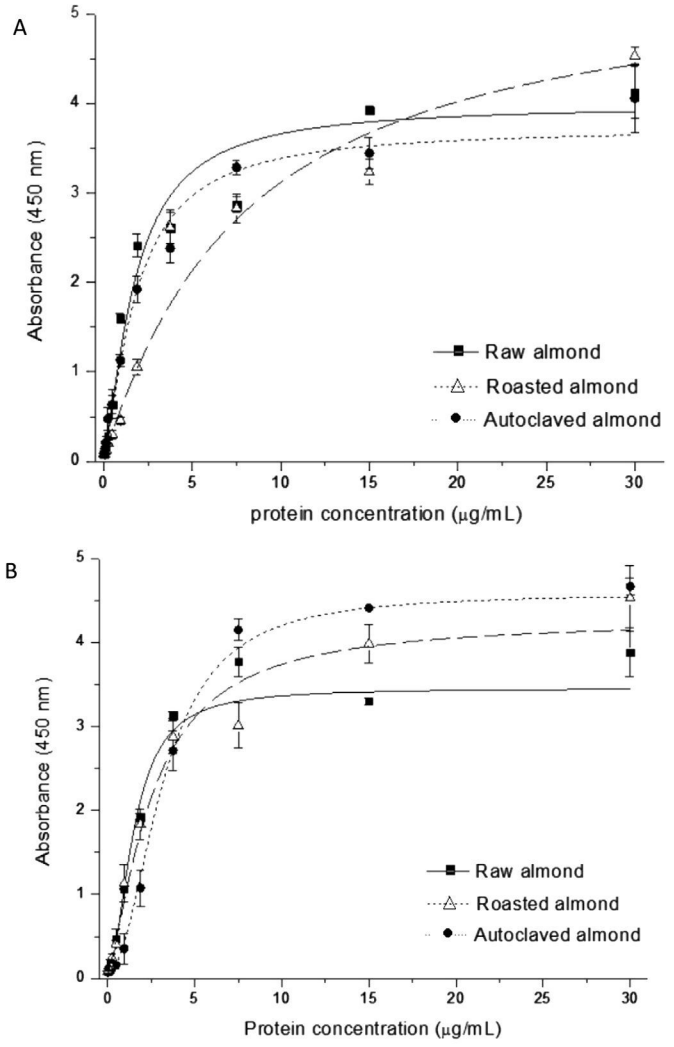

Fig. 3. Representative standard curves of the developed almond phage ELISA. Each curve shows the average value of six independent experiments and the standard deviation in each point of the curve. (A) Clone PD1F6 against dilutions of raw, roasted and autoclaved almond protein extracts. (B) Clone PD2C9 against dilutions of raw, roasted and autoclaved almond protein extracts (see Section 2.10 for details).

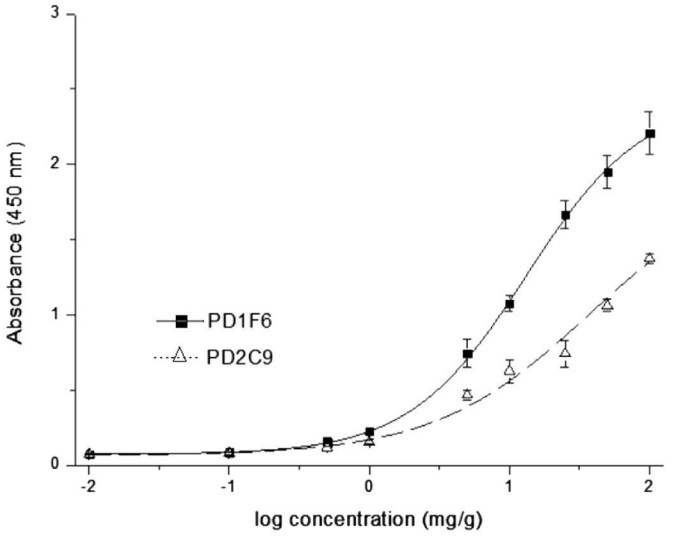

Fig. 4. Representative standard curves of the almond phage ELISA performed with protein extracts obtained from almond/wheat flour binary mixtures. Each curve shows the average value of six independent experiments and the standard deviation in each point of the curve.

increase in thermostability when encountered in low-water systems such whole food matrices. Therefore, heating almond proteins in solution can enhance denaturation of proteins. To confirm that hypothesis, we heated the protein extracts at 100 °C for 1, 5 and 10 min, and we observed a lack of response in the ELISA after heating for 5 and 10 min. These results differ from those obtained when roasting or autoclaving ground or whole almond kernels, as the protein extracts obtained from the heat treated almonds were perfectly detected by the almond-specific phage-antibodies. Therefore, the presence of high water content during the thermal processing of the samples might have led to irreversible denaturation of the epitopes recognized by the phages. Dhakal et al., 2014 also found a reduction in immunoreactivity of an almond protein when heating samples at temperatures above 85 °C for 5 min.

Concerning the samples that declared traces of almond, just 1 out of 16 chocolates analyzed showed positive results, and the only biscuit sample analyzed was negative. Those results were supported by the real-time PCR. Frequently almond containing products are produced in factories along with other products non-containing almond. In such scenario, cross-contamination problems with allergens might happen, so companies prefer warning allergic consumers against the possible presence of the allergen, even when the product actually would not contain almond. However, misuse of advisory labeling nullifies its objectives, because it might reduce the range of products available to allergic consumers, leading to dietary restriction, frustration and reckless behavior. Labeling alerts on possible cross-contamination with allergens is justifiable only on the basis of a risk analysis applied to a responsibly managed operation (Pérez, J. S. 2013). Therefore, it is still necessary to develop approaches for the application of advisory labeling.

Finally, in the products analyzed that declared to contain traces of tree nuts and also those that did not declare to contain nuts, we observed discrepancies in some samples between almond ELISA and real-time PCR results. However, it should be noted that the real-time PCR method is able to detect almond concentrations down to 0.1 ppm. In those products that were negative in ELISA but positive in real-time PCR, the Cp values obtained were above 35, that when substituted in the corresponding regression line (López-Calleja et al., 2014), means a concentration value of about 30 ppm, which is below the LOD achieved with the almond phage-ELISA (110 ppm).

Although the threshold dose for an allergen to elicit an allergic response varies within the allergen and the individual (Taylor et al., 2002), the general consensus is that an adequate food allergen detection method requires enough sensitivity to detect amounts of the target protein at levels as low as 1–100 ppm (Wensing et al., 2002). Thus the ELISA method developed in this work combines high specificity and easy-to-use methodology, and the LOD of detection achieved is in the upper limit of those considered appropriate to protect allergic consumers from undeclared almond in food products.

Almond detection assays published in literature mainly entail almond DNA identification by real-time PCR (Costa, Oliveira, & Mafra, 2013; Köppel et al., 2009; López-Calleja et al., 2014; Röder, Vieths, & Holzhauser, 2011) or protein detection by immunochemical methods (Acosta et al., 1999; Ben Rejeb et al., 2005; Hlywka et al., 2000; Su et al., 2013). Immunochemical methods developed to date have employed polyclonal or monoclonal antibodies raised in animals to report the presence of the target molecule. Some of these methods, in spite of demonstrating

Table 3
Determination of the presence of almond in various commercial processed food products using almond phage-ELISA and real-time PCR.

Label statement	Product	Number of samples analyzed	Almond phage ELISA[a]	ITS real-time PCR[a]
Almond declared as ingredient	nut bar	4	+(4)	+(4)
	breakfast cereals	9	+(9)	+(9)
	chocolate	2	−(2)	−(2)
	beverage	2	−(2)	+(2)
Contains traces of almonds	chocolate	16	+(1)/−(15)	+(1)/−(15)
	biscuit	1	−(1)	−(1)
May contain traces of tree nuts	biscuit	11	−(11)	+(3)[b]/−(8)
	food bar	10	−(10)	+(3)[b]/−(7)
	chocolate	8	Traces (2)/−(6)	+(4)[b]/−(4)
	sauce	1	−(1)	−(1)
	cashew butter	1	−(1)	−(1)
	bread	3	−(3)	+(3)[b]
	beverage	3	−(3)	+(2)[b]/−(1)
	breakfast cereal	2	−(2)	+(1)[b]/−(1)
	chocolate cream	1	Traces (1)	+(1)[b]
	precooked meal	1	−(1)	+(1)[b]
	ice cream	2	−(2)	+(1)[b]/−(1)
Not declaring to contain nuts or traces	chocolate	1	−(1)	−(1)
	beverage	3	−(3)	+(2)[b]/−(1)
	sesame bar	1	Traces (1)	+(1)[b]
	ice cream	1	−(1)	−(1)
	biscuit	4	−(4)	−(4)
	chocolate cream	1	−(1)	−(1)
	bread	2	−(2)	+(1)[b]/−(1)
	powdered infant cereals	2	−(2)	−(2)

[a] A plus (+) indicates absorbance values above the LOD (ELISA) or the presence of amplification after 50 cycles (real-time PCR), and a minus (−) indicates absorbance values lower that LOD or an absence of amplification after 50 cycles (real-time PCR).
[b] Cp values >35, corresponding to almond concentration lower than 30 ppm.

adequate sensitivity, show cross-reactivity problems with several tree nuts and other ingredients (Ben Rejeb et al., 2005; Hlywka et al., 2000), immunoreactivity failure after heat treatments (Acosta et al., 1999), or the drawback of variability attributed to the nature of polyclonal antibodies employed at any stage of the ELISA. The main goal of the phage-ELISA developed herein is the use of recombinant antibody fragments isolated from a phage display library instead of traditional antibodies, taking into account the advantages of using bacterial expression systems, like speed and economy. However, unlike traditional antibodies, that undergo a natural affinity maturation process, recombinant antibody technology requires artificial affinity maturation to improve the affinity of the antibody candidate (Renaut et al., 2012).

The purpose of our research was to demonstrate that it was possible (1) to isolate a specific scFv against almond protein from a naïve commercial phage display library, (2) by employing a whole protein extract instead of a purified protein, (3) and that the isolated scFv could be employed in an ELISA to detect almond protein when displayed fused to the PIII protein of a filamentous phage. The selected phage antibodies are highly specific for detection of almond in food products, and as a stable biological reagent, their sensitivity can be further improved by affinity maturation. Furthermore, the scFv can be genetically engineered with a large diversity of expression tags such as enzymes and fluorescent probes that could be used in a myriad of assay formats.

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